

Development of Andrographolide-Carboxymethyl Chitosan Nanoparticles: Characterization, in vitro Release and in vivo Antimalarial Activity Study

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Andrografolid Karboksimetil Kitosan Nanopartiküllerinin Geliştirilmesi: Karakterizasyon, *in vitro* Salım ve *in vivo* Antimalaryal Aktivite Çalışması

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ABSTRACT

Objectives: The purpose of this study was to investigate the effect of andrographolide-carboxymethyl chitosan nanoparticles formation on the physical characteristics, *in vitro* release profile and *in vivo* antimalarial activity of andrographolide.

Materials and Methods: Nanoparticles were prepared by ionic gelation method-spray drying using CaCl_2 as the crosslinker with a composition of drug: polymer $\text{CaCl}_2=40: 250: 100$. The obtained particles were evaluated for its size and morphology; physical state, drug content, *in vitro* drug release and *in vivo* antimalarial activity on *Plasmodium berghei* infected mice.

Results: The results of DTA and XRD showed that nanoparticle systems had a lower melting point and lower crystallinity degree. The drug dissolved from the nanoparticles was increased up to 6.5 times and the *in vivo* antimalarial activity was 1.65 times higher compared to andrographolide.

Conclusion: The formation andrographolide-carboxymethyl chitosan nanoparticles affected the physical characteristics of andrographolide. The decrease crystallinity of andrographolide resulted in a lower melting point of andrographolide. Such changes provided a positive impact to the drug dissolution and then its activity.

Key words: Andrographolide, carboxymethyl chitosan, *in vitro* release, *in vivo* antimalarial, ionic gelation, spray drying

ÖZ

Amaç: Bu çalışmanın amacı, andrografolid-karboksimetil kitosan nanopartiküllerinin oluşumunun andrografolidin fiziksel özellikleri, *in vitro* salım profili ve *in vivo* antimalaryal aktivitesi üzerine etkisini araştırmaktır.

Gereç ve Yöntemler: Nanopartiküller iyonik jelasyon yöntemi-püskürtürerek kurutma ile çapraz bağlayıcı olarak CaCl_2 kullanılarak etken madde: polimer $\text{CaCl}_2=40: 250: 100$ bileşimi ile hazırlandı. Elde edilen partiküller büyüklükleri ve morfolojisi, fiziksel durumu, etken madde içeriği, *in vitro* etken madde salımı ve *Plasmodium berghei* ile enfekte sıçanlarda *in vivo* antimalaryal aktivite açısından değerlendirildi.

Bulgular: DTA ve XRD sonuçları, nanopartikül sistemlerinin daha düşük bir erime noktasına ve daha düşük kristallik derecesine sahip olduğunu gösterdi. Nanopartiküllerden çözünen etken madde 6.5 kat artmıştır ve *in vivo* antimalaryal etkinlik andrografolid ile karşılaştırıldığında 1.65 kat daha fazladır.

Sonuç: Andrografolid-karboksimetil kitosan nanopartiküllerinin oluşumu andrografolidin fiziksel özelliklerini etkiledi. Andrografolidin kristalinitesinin azalması, andrografolidin erime noktasının daha düşük olmasına neden olmuştur. Bu değişiklikler, etken maddenin çözünmesine ve daha sonra etkinliğine olumlu bir etki sağlamıştır.

Anahtar kelimeler: Andrografolid, karboksimetil kitosan, *in vitro* salım, *in vivo* antimalaryal, iyonik jelleşme, püskürtürerek kurutma

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INTRODUCTION

According to the World Health Organization report 2014, 17.1% of all essential medicines are classified as BCS II (high permeability and low solubility) and 10.6% of them are classified as BCS IV (low permeability and low solubility). A drug can be absorbed by the body and provide a pharmacologic effect for the body if the drug in a dissolved state.¹ Thus, a low solubility drug results in low bioavailability. Andrographolide, a diterpene lactone obtained from *Andrographis paniculata*, has extensive pharmacologic effects such as anti-inflammatory, anti-diarrhea, anti-HIV, anti-malarial, hepatoprotective, anticancer, antioxidant, and antihypertensive properties. Andrographolide application is restricted due to its low water solubility, short half-life time (2 hours) and low permeability. Pharmacokinetic studies showed that andrographolide was quickly absorbed and metabolized in rats and humans.^{2,3} Several methods have been used to improve the low solubility of andrographolide. These methods are chemical modification, solid dispersion, liposomes, and nanoparticles. By improving the solubility, it is expected to increase the release rate and bioavailability.^{2,4} Formation of nanoparticles andrographolide-eudragit® EPO increased bioavailability 2.2 times compared with pure andrographolide in oral administration.⁵ Nanoparticles are a dispersion of solid particles with a diameter ranging between 10-1000 nm consisting of drug which is dispersed, trapped or enveloped in a matrix of nanoparticles. Nanoparticles can be used as a drug carrier in drug therapy or vaccine adjuvant, and because of their small size, they can increase the absorption of the drug into the biologic membrane by facilitated diffusion.⁶⁻⁸

Carboxymethyl chitosan is a derivative of the water-soluble chitosan. Having amine and carboxyl groups in the molecule enables it to be used as a carrier in drug delivery systems because these materials are biocompatible, biodegradable, and non-toxic.⁹ Carboxymethyl chitosan nanoparticles can be prepared by emulsification, gelation ionic, coacervation, spray drying, sonication, emulsion droplet-coalescence, reverse micellar, and sieving. The mechanism of formation of carboxymethyl chitosan nanoparticles with an ionic gelation method is based on the electrostatic interaction between carboxyl groups of carboxymethyl chitosan and a positive charge of the crosslinker, CaCl_2 . The addition of CaCl_2 is intended to form a bond between the divalent cations Ca^{2+} ions and CaCl_2 with $-\text{COO}^-$ of carboxymethyl chitosan.^{9,10}

Malaria is an infectious disease caused by the parasite *Plasmodium*, which is characterized by fever, anemia, and splenomegaly.¹¹ There were 183 million cases of malaria and 584 thousand deaths (78% children under 5 years of age) due to malaria worldwide by 2013. Resistance to antimalarial drugs such as chloroquine and sulfadoxine-pyrimethamine is a problem that results in increased morbidity and mortality.¹² Therefore, the development of antimalarial drug delivery systems still needs to be achieved. In the present study, carboxymethyl chitosan nanoparticles of andrographolide were made by ionic gelation-spray drying to improve the physical properties and antimalarial activity of andrographolide. The particles obtained were evaluated for their morphology, physical state, *in vitro*

release, and *in vivo* antimalarial activity on *Plasmodium berghei*-infected mice.

EXPERIMENTAL

Material

Andrographolide (RD Health Ingredients Co., Ltd.); Carboxymethyl chitosan (degree of substitution 81.9%, 96.5% degree of deacetylation, viscosity 1% 22 mPa.s, China Eastar Group Co., Ltd.); Calcium chloride $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ pro analysis (Merck); methanol; 96% ethanol pro analysis; distilled water; *Plasmodium berghei* ANKA strain obtained from the Eijkman Institute for Molecular Biology, Jakarta and maintained SATREPS ITD (Institute of Tropical Disease); Alceivers medium; Giemsa dye in phosphate buffer; absolute methanol.

Preparation of andrographolide-carboxymethyl chitosan nanoparticles

The andrographolide-carboxymethyl chitosan nanoparticles were prepared by ionic gelation using CaCl_2 as a crosslinker then spray dried. Two hundred milligrams of carboxymethyl chitosan were dissolved in 100 mL of distilled water. Carboxymethyl chitosan solution was then poured into andrographolide solution (40 mg in 5 mL methanol) and stirred briefly at 500 rpm. The andrographolide-carboxymethyl chitosan solution was added into CaCl_2 solution (100 mg in 40 mL ethanol-water=1:9) and the mixture solution was stirred constantly for 4 hours at 500 rpm. Non-crosslinked particles were also prepared through simple mixing of the polymer solution and andrographolide solution. Dry particles were obtained by spray drying using SD Basic, LabPlant with 1.0 mm nozzle diameter at inlet temperature 100°C, flow rate 5 mL/min and pressure 2 mBar.

Particle morphology and size evaluation

The size, shape, and surface morphology of the particles were observed using scanning electron microscopy. Particles were embedded in a holder made of aluminum and coated with gold palladium prior to analysis. Pictures were taken at various magnifications at 20.00 kV.

Fourier transform infrared (FT-IR)

Particles were made as a pellet by mixing with KBr powder then pressed with a hydraulic pump to form a transparent pellet. Sample observation was conducted at wavelength 4000-450 cm^{-1} (Jasco FT-IR 5300, Easton MD, USA).

Differential thermal analysis (DTA)

Thermal analysis of the sample was conducted using a differential thermal analyzer (DTA FP-65 P-900 Thermal, Mettler Toledo, USA). About 5 mg of particles were placed in a crucible pan, sealed and observed for a thermogram. The thermogram was recorded at temperatures of 50 to 250°C with a heating rate 10°C/min.

X-ray diffractometry

X-ray diffraction analysis was conducted using a Phillips X'Pert X-ray diffractometer to evaluate the samples' crystallinity.

The light source employed was a $K\alpha$ Cu Ni. The voltage and the current were set at 40 kV and 40 mA. Samples were analyzed at 2θ and angle between $5-40^\circ$. The diffractogram of the andrographolide-carboxymethyl chitosan particles was compared with the diffractogram of base andrographolide.

Drug content

The andrographolide content in nanoparticle was determined using high-performance liquid chromatography (HPLC) Agilent 1100 Series using reverse phase with a mobile phase consisting of methanol-orthophosphoric acid (50:50, pH 3) at a wavelength of 228 nm. Five milligrams of accurately weighed particles were dissolved in 10.0 mL of methanol, filtered through a $0.2\text{-}\mu\text{m}$ membrane filter, and then the 7 mL sample was injected into the HPLC column. The assay is performed in triplicate.

The drug content was calculated using the equation below:

$$\% \text{ Drug content} = \frac{\text{drug amount}}{\text{particle weight}} \times 100\%$$

In vitro release study

The *in vitro* release study was performed in 50 mL 0.1% w/v sodium lauryl sulfate (SLS) media at $37 \pm 0.5^\circ\text{C}$, 120 rpm using a water bath shaker. SLS was used to improve the dissolution of andrographolide.¹³ Andrographolide-carboxymethyl chitosan particles equivalent to 2 mg of andrographolide were weighed accurately and put into the media. A 1.0-mL sample was taken at a predetermined time for 3 hours and the same volume of media was added after sampling. Andrographolide was also evaluated as control. The samples were analyzed using HPLC as described above. This evaluation was performed triplicate.

In vivo antimalarial activity test

The *in vivo* antimalarial activity test was conducted according to the method of Peter. The 4-day suppressive test of blood schizonticidal action.¹⁴ Male mice aged 1.5-2 months of the strain Balb/C weighing 20-30 grams were supplied by the Department of Parasitology, Faculty of Medicine, University of Brawijaya, Malang. All mice were acclimatized in the animal house and were fed with standard diet and water *ad libitum*. The use of animals in this study was approved by the Animal Care and Use Committee (ACUC) of the Veterinary Faculty, Airlangga University (591-KE). All mice were injected intraperitoneally with 200 μL *Plasmodium berghei* infected mice's blood, which contained approximately 10^5 parasitized erythrocytes. Sixteen mice were used and were divided into four groups. Group 1 and group 2 were orally treated twice daily for four days with andrographolide suspended in carboxymethyl chitosan solution, and andrographolide-carboxymethyl chitosan particles suspended in water, respectively, with doses equivalent to 12.5 mg andrographolide/kg. Group 3 received carboxymethyl chitosan solution and group 4, as the untreated/control group, received water. Over a period of 6 days, tail blood was withdrawn and parasitemia was monitored by examining Giemsa-stained thin blood smears using an optical microscope. Parasitemia was calculated using the following equation:

$$\% \text{ parasitemia} = \frac{\text{number of parasitized erythrocytes}}{\text{number of erythrocytes}} \times 100\%$$

Percentage of parasitemia inhibition was calculated on day 5 using equation below.

$$\% \text{ inhibition} = 100\% - \frac{\% \text{ parasitemia growth of treated group}}{\% \text{ parasitemia growth of untreated group}} \times 100\%$$

RESULT AND DISCUSSION

Figure 1 shows the morphology of andrographolide-carboxymethyl chitosan nanoparticles and non-cross-linked andrographolide-carboxymethyl chitosan. Cross-linked andrographolide-carboxymethyl chitosan was seen as non-spherical with a hollow shape in a micrograph, whereas the non-cross-linked andrographolide-carboxymethyl chitosan produced more spherical and smooth particles. The andrographolide crystal was observed in cross-linked particles. It indicated that andrographolide was solidified thus hindered the formation of spherical and smooth particles shape. Cross-linked andrographolide-carboxymethyl chitosan nanoparticles had a range of sizes from 600 nm to 3000 nm, whereas the non-cross-linked andrographolide-carboxymethyl chitosan particle sizes were within the range of 500 nm to 2500 nm.

DTA

From the DTA thermogram in Figure 2, it was identified that andrographolide had a melting range 231.6°C with a sharp

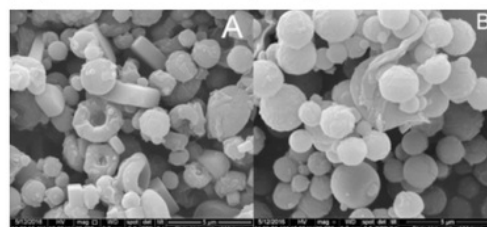


Figure 1. SSEM of crosslinked andrographolide-chitosan particles (A) and non crosslinked andrographolide-chitosan particles (B) (magnification 20000x)

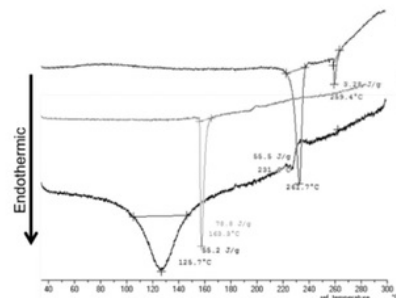


Figure 2. DTA thermogram of andrographolide (A), and non-crosslinked andrographolide-chitosan particles (B), crosslinked andrographolide-chitosan particles (C)

endothermic peak, and non-cross-linked andrographolide-carboxymethyl chitosan particles had a sharp endothermic peak with melting point of 162.9°C. The thermogram of andrographolide-carboxymethyl chitosan nanoparticles shifted to the lower melting point of 125.7°C with a wider endothermic peak compared with andrographolide and the non-cross-linked andrographolide-carboxymethyl chitosan particles. It indicated that the formation of cross-linked particles resulted in lower ordered structural molecule patterns compared with the non-cross-linked particles and base andrographolide.

X-ray diffractometry

As shown in Figure 3, the diffractogram of andrographolide indicated crystalline peaks with high intensity on 2θ 9.83°, 14.81°, 15.69°, 15.85°. CaCl_2 itself had a diffraction peak at 14.74° 2θ . In the diffractogram of andrographolide-carboxymethyl chitosan physical mixture, several crystalline peaks with low intensity at 12.14°, 15.77° and 18.55° were detected.

The diffractogram of andrographolide-carboxymethyl chitosan nanoparticles showed that diffraction peaks of andrographolide, carboxymethyl chitosan, and CaCl_2 disappeared, but new crystalline peaks at 31.63° 2θ appeared. These results showed that the formation of cross-linked particles of andrographolide-carboxymethyl chitosan prepared by ionic gelation-spray drying had lower crystallinity compared with andrographolide itself.

Drug content

From the drug content evaluation using HPLC, the andrographolide content in the particles was found as $12.09 \pm 0.26\%$. The result was further used to calculate the

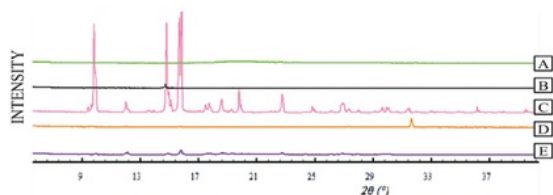


Figure 3. X-ray diffractogram of (A) carboxymethyl chitosan, (B) CaCl_2 , (C) andrographolide (D) crosslinked andrographolide-chitosan particles, dan (E) physical mixture of andrographolide-chitosan

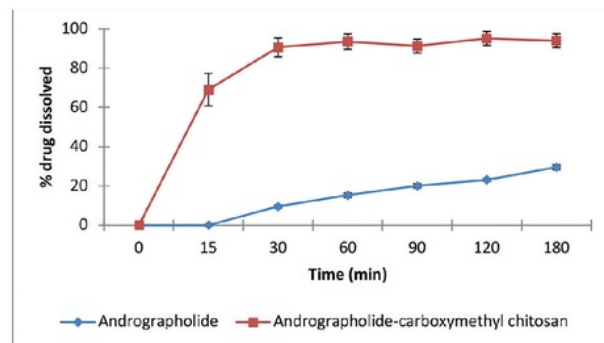


Figure 4. *In vitro* release profile of andrographolide-carboxymethyl chitosan particles (D) and crosslinked andrographolide in 0.1% SLS media at $37 \pm 0.5^\circ\text{C}$ ($n=3$)

amount of andrographolide-carboxymethyl chitosan for the *in vitro* release test and *in vivo* antimalarial activity test.

In vitro release study

The *in vitro* release test was performed in 0.1% SLS to facilitate drug dissolution. The result demonstrated that the amount of andrographolide dissolved in 15 min from the nanoparticle systems (69.06%) was greater compared with andrographolide. After 30 min, the amount of andrographolide dissolved from the nanoparticles was up to 10 times higher than with base andrographolide (Figure 4).

The slope that indicated the release rate of andrographolide from carboxymethyl particles and base andrographolide were $12.7158 \pm 0.8054\%$ dissolved/ $\text{min}^{1/2}$ and $2.0221 \pm 0.2702\%$ dissolved/ $\text{min}^{1/2}$, respectively. The result indicated that the formation of andrographolide-carboxymethyl chitosan could increase the release rate of andrographolide 6.3 times compared with andrographolide itself. This was due to the formation of particles prepared by ionic gelation-spray drying lead to changes the crystallinity of the drug as well as has being shown by the results of the DTA thermogram and X-ray diffractogram in Figure 2 and Figure 3. The entrapment of andrographolide into the cross-linked carboxymethyl chitosan continued with fast solidification of the particles during the spray drying process, which caused inhibition of crystal growth, could result in an amorphous form and crystal size reduction of andrographolide. The changes of crystallinity structure into the amorphous form and reduction of particle size of poorly soluble drug would be advantageous because it would enhance the solubility and then its bioavailability.^{15,16}

In vivo antimalarial activity test

Figure 5 shows that parasitemia growth occurred during the evaluation in all groups. The treated groups led to a slow growth of parasitemia because the untreated/control group

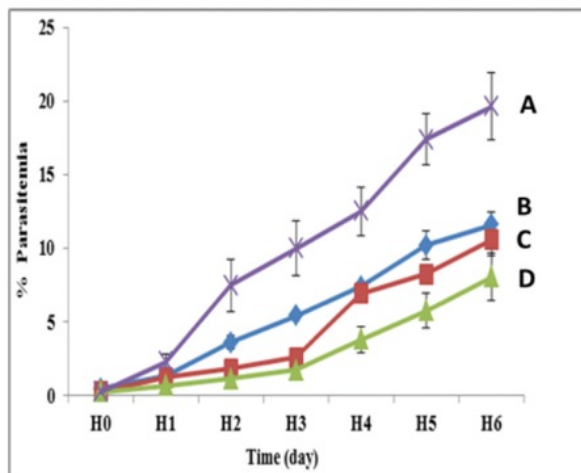


Figure 5. Parasitaemia growth curve of *Plasmodium berghei* infected mice of (A) control group, (B) andrographolide treated group, (C) carboxymethyl chitosan treated group and (D) andrographolide-carboxymethyl chitosan particles treated group

had rapid growth parasitemia. The antimalarial activity test results presented in Figure 5 and Figure 6 revealed that the increasing number of *Plasmodium berghei* infected erythrocytes (parasitaemia) were lower than the control/untreated group in all treated groups.

The growth inhibition of parasitemia on day five of andrographolide system-carboxymethyl chitosan nanoparticles-treated group was $71.27 \pm 6.83\%$, which was higher compared with the andrographolide-treated group ($43.30 \pm 1.83\%$) and carboxymethyl chitosan-treated group ($45.48 \pm 3.71\%$). The formation of andrographolide nanoparticles with carboxymethyl chitosan changed the physical state of andrographolide, which lowered its melting point and degree of crystallinity, as shown in Figure 2 and Figure 3. These results will improve the dissolution of andrographolide and further, will provide a favorable effect on its activity. The parasitemia growth inhibition of the andrographolide-carboxymethyl chitosan particles increased 1.65 times compared with andrographolide and was statistically significantly different (Table 1).

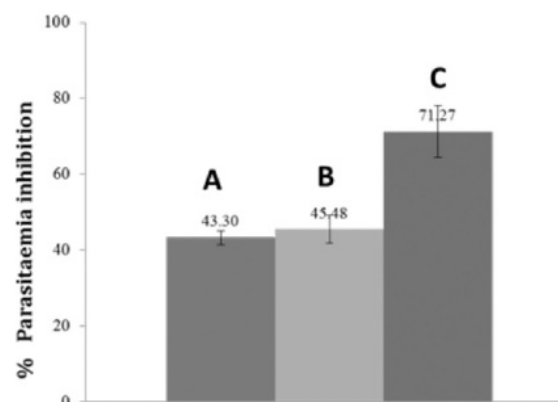


Figure 6. Histogram of parasitaemia inhibition percentage of *Plasmodium berghei* infected mice of (A) andrographolide treated group, (B) carboxymethyl chitosan treated group and (C) andrographolide-carboxymethyl chitosan particles treated group

Table 1. One-way ANOVA ($p=0.05$) to determine the effect of andrographolide, carboxymethyl chitosan and andrographolide-carboxymethyl chitosan particles on parasitaemia inhibition of *O* infected mice

Group	n	% Parasitaemia inhibition \pm SD	ANOVA	
			Result	Conclusion
Andrographolide treated group	4	43.30 ± 1.83^a	F=11.373 p=0.002	Significantly different
Carboxymethyl chitosan treated group	4	45.48 ± 3.71^a		
Andrographolide-carboxymethyl chitosan particles treated group	4	71.27 ± 6.83^b		

Note: ^{a,b} Signs refer to no difference between the groups. n: Sample number

CONCLUSION

The formation system of andrographolide-carboxymethyl chitosan nanoparticles affected the physical characteristics of andrographolide. The crystallinity decrease of andrographolide resulted in a lower melting point of andrographolide. Such changes had a positive impact on the drug dissolution and then its activity. The release rate of andrographolide from carboxymethyl chitosan nanoparticles increased up to 6.3 times and *in vivo* antimalarial activity in *Plasmodium berghei*-infected mice was significantly enhanced up to 1.65 times compared with base andrographolide.

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Conflict of Interest: No conflict of interest was declared by the authors.

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